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Purification of 11 β -hydroxysteroid dehydrogenase type 2 from human placenta utilizing a novel affinity labelling technique

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11 β -Hydroxysteroid dehydrogenase type 2 (11 β -HSD2) efficiently inactivates potent glucocorticoid hormones (cortisol and corticosterone), leaving aldosterone unmetabolized. Abundant 11 β -HSD2 activity in human placenta plays a central role in controlling fetal glucocorticoid exposure, which if excessive is harmful and may predispose to low birth weight and hypertension in adulthood. Similar 11 β -HSD2 activity in the distal nephron protects mineralocorticoid receptors from glucocorticoids and appears to be important in normal blood pressure control. We have purified human placental 11 β -HSD2 16000-fold, to homogeneity, and determined over 100 residues of the internal amino acid sequence. Purification was assisted by a novel

technique allowing highly specific (single spot on two-dimensional electrophoresis) photoaffinity labelling of active 11 β -HSD2 in crude tissue extracts by its glucocorticoid substrates. This work reveals that 11 β -HSD2 is a member of the short-chain alcohol dehydrogenase superfamily (apparent monomer $M_r \sim 40000$). It is a very basic (apparent pI = 9.1) intrinsic membrane protein, requiring as yet undefined membrane constituents for full stability. Affinity chromatography and affinity labelling studies suggest that 11 β -HSD2 has a compulsory ordered mechanism, with NAD⁺ binding first, followed by a conformational change allowing glucocorticoid binding with high affinity.

INTRODUCTION

11 β -Hydroxysteroid dehydrogenase (11 β -HSD) catalyses the rapid metabolism of physiological glucocorticoids (cortisol and corticosterone) to inactive 11-dehydro products (cortisone and 11-dehydrocorticosterone respectively). In any tissue the activity of 11 β -HSD will affect the amount of circulating glucocorticoids reaching their intracellular receptors [glucocorticoid receptors (GR) and mineralocorticoid receptors (MR)]. Moreover, as the mineralocorticoid aldosterone is not metabolized by 11 β -HSD, it can gain selective access to the MR in the face of 100–1000-fold higher levels of circulating glucocorticoids, providing that 11 β -HSD activity is sufficient to eradicate the glucocorticoid. It is now widely believed that just such a selective barrier to glucocorticoid is present in aldosterone target tissues [1,2] (e.g. the distal nephron in kidney) and enables potent effects on fluid and electrolyte balance and blood pressure to be exerted by aldosterone acting through MR (which *in vitro* bind aldosterone and physiological glucocorticoids with equal affinity [3]). Deficiency of 11 β -HSD due either to the congenital syndrome of apparent mineralocorticoid excess (SAME) [4,5] or to the ingestion of 11 β -HSD inhibitors [6] (liquorice constituents or carbenoxolone) leads to the normally protected tissues being accessed by glucocorticoids, which occupy MR and cause mineralocorticoid hypertension.

There is also abundant 11 β -HSD activity in the placenta which has a major influence on the glucocorticoid exposure of the developing fetus [7–10]. Glucocorticoids are important in normal development, facilitating the maturation of tissues (e.g. lung [11]) and influencing the set point of aspects of fetal physiology, a number of which then become fixed for life (fetal programming) [12–15]. If the fetus is exposed to excessive glucocorticoids, birth weight is reduced [9,16] and, in animal models, the offspring are hypertensive in adulthood [9]. Inhibition of placental 11 β -HSD

similarly reduces birth weight and results in hypertensive offspring (R. S. Lindsay, R. M. Lindsay, C. R. W. Edwards and J. R. Seckl, unpublished work). Human epidemiology also reveals that those babies with the lowest birth weight have the greatest risk of adult hypertension [17]. Furthermore, in rats [9] and humans [10], birth weight correlates with placental 11 β -HSD activity.

The previously identified isoform of 11 β -HSD (11 β -HSD1) is not responsible for the renal and placental barriers to glucocorticoids. It has been purified from rat liver [18], and corresponding rat [19], human [20], squirrel monkey [21], sheep [22] and mouse [23,24] cDNA clones have been isolated. 11 β -HSD1 is a reversible, NADP(H)-dependent enzyme, with high expression in some tissues (e.g. liver), but not in the distal nephron or placenta, and it has relatively low affinity for glucocorticoids (K_m in the low micromolar region) [18]. Moreover, no mutations of the 11 β -HSD1 gene have been identified in SAME patients [25]. A distinct NAD⁺-dependent, exclusively dehydrogenase, 11 β -HSD activity with high affinity for glucocorticoids has been identified in biochemical studies in rabbit kidney cortical collecting duct cells [26] and human placenta [27]. Similar activity has now been described in renal tissue of other species [28] and several human fetal tissues [29]. These activities are due to a distinct isoform, 11 β -HSD2 (or possibly several closely related ones), and seem likely to be responsible for the important barriers to glucocorticoid access described above. Further study of 11 β -HSD2 has been hampered by the lack of specific ‘molecular tools’ to investigate it at the protein and nucleic acid levels.

In this paper we present the first purification of 11 β -HSD2 to homogeneity and an extensive amino acid sequence derived directly therefrom. We also describe a novel affinity labelling procedure allowing 11 β -HSD2 to be specifically labelled by its natural substrates. Aspects of this work should facilitate study of the mechanism of action of 11 β -HSD2 and the mapping of its

Abbreviations used: 11 β -HSD(2), 11 β -hydroxysteroid dehydrogenase (type 2); GR, glucocorticoid receptors; MR, mineralocorticoid receptors; SAME, syndrome of apparent mineralocorticoid excess; CAPS, 3-(cyclohexylamino)-1-propanesulphonic acid; 2-D, two-dimensional; CMC, critical micelle concentration; IEF, isoelectric focusing; NEPHGE, non-equilibrium pH-gradient gel electrophoresis; TFA, trifluoroacetic acid; SCAD, short-chain alcohol dehydrogenase; PVDF, poly(vinylidene difluoride).

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active site. Such study will be valuable in the efficient design of new drugs influencing corticosteroid physiology (e.g. by selectively inhibiting, bypassing or being metabolized effectively by this key enzyme). In an accompanying paper we report use of the 11 β -HSD2 amino acid sequence to isolate and characterize a full-length human placental 11 β -HSD2 cDNA clone and to raise antisera to the placental 11 β -HSD2 protein [30].

EXPERIMENTAL

Materials

[1,2,6,7-³H]Corticosterone, [1,2,6,7-³H]cortisol, [1,2,4,6,7-³H]dexamethasone and [1,2,6,7-³H]aldosterone (specific radioactivity 78, 73, 84 and 80 Ci/mmol respectively) were obtained from Amersham International (Little Chalfont, Bucks., U.K.). Autodigestion-resistant modified trypsin and *Staphylococcus aureus* V8 protease were obtained from Promega (Southampton, U.K.), and hydrogenated Triton X-100 (RTX-100; protein grade) was from Calbiochem (Nottingham, U.K.). Glycerol and protein standards for SDS/PAGE (#44264L), electrophoresis-grade SDS and urea were purchased from BDH Laboratory Supplies (Poole, Dorset, U.K.). Coomassie Blue dye concentrate, standardized BSA and protein standards for two-dimensional (2-D) electrophoresis (#1610320) were purchased from Bio-Rad (Hemel Hempstead, U.K.). HPLC-grade methanol and water were purchased from Rathburn Chemicals (Walkerburn, Scotland, U.K.) and Quickszint 302 HPLC scintillant was from Zinsser Analytic (Maidenhead, U.K.). Intensify fluoroautoradiography solutions were obtained from du Pont/NEN (Stevenage, U.K.). Poly(vinylidene difluoride) (PVDF; Problott) membranes were obtained from Applied Biosystems (Warrington, U.K.). Affinity chromatography matrices, Pharmalytes, detergents and other chemicals were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

Buffers

Chromatography buffer systems used were as follows. Buffer A: 20% glycerol, 5 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol. Buffer B: 10% glycerol, 300 mM NaCl, 4 mM CHAPS, 1 mM EDTA, 0.02 M Tris/HCl, pH 7.7. Buffer C: 10% glycerol, 300 mM NaCl, 1 mM EDTA, 0.02 M Tris/HCl, pH 7.7. Where the buffer varies from that above, the variation is appended in [brackets].

Subcellular fractionation of placental tissue

Human term placentae (400–600 g; normal vaginal delivery) were rapidly placed on ice, and tissue was processed within 2 h. Adherent membranes, umbilical cord and large vessels were removed. Placental tissues were rapidly minced with scissors, washed in ice-cold 0.9% NaCl, blotted dry, suspended in approximately 3 times their weight of buffer A and homogenized with a commercial blender. Homogenate was filtered through two layers of muslin and the filtrate fractionated rapidly by differential centrifugation: (i) 10 min at 750 g, (ii) 40 min at 25000 g and (iii) 60 min at 110000 g. The supernatant from each centrifugation was subjected to the next centrifugation, finally leaving a cytosolic supernatant. Resulting fractions were frozen at –80 °C or used immediately.

Assays of 11 β -HSD activity

11 β -HSD2 activity was determined by measuring the percentage conversion of 1.12×10^{-8} M ³H-labelled steroid substrate (cortico-

sterone unless otherwise stated) to product (11-dehydrocorticosterone) in the presence of NAD⁺ (400 μ M unless otherwise stated). The 250 μ l assay consisted of 10 μ l containing tritiated steroid, 50 μ l containing cofactor and 190 μ l of enzyme in buffer C. Reactions were incubated at 37 °C for 10 min and terminated by adding 2 ml of ethyl acetate. The steroids (in the organic layer) were assayed by HPLC as previously described [27], and the percentage conversion of steroid substrate to product was calculated as an index of enzyme activity. Protein was estimated by the method of Bradford [31]. 11 β -HSD assay conditions were such that the amount of protein added was in the linear region of the curve of protein concentration versus percentage substrate converted with 400 μ M NAD⁺, and resulted in conversion of 10–40% in 10 min. All experiments had blank (no protein) assays run in parallel. Kinetic parameters were calculated from initial-velocity determinations in assays giving less than 30% conversion.

Detergent solubilization

All solubilization was carried out at 0–2 °C. Tissue fractions, resuspended in buffer C to 6 mg of protein/ml, were mixed with an equal volume of solubilization buffer (in buffer C with detergent at twice the final concentration). After 30 min the mixture was centrifuged at 110000 g for 1 h. Supernatant containing soluble enzyme was carefully removed. A screen was performed using 13 detergents of diverse classes [32] (Triton DF18 [18], CHAPS, taurodeoxycholic acid, polyoxyethylene-10-lauryl ether, digitonin, n-octyl glucoside, Tween 20, Tween 80, SDS, n-lauryl sarcosine, Triton X-100, Zwittergent 3-10 and Bigchaps). Following this screen, conditions were refined for the most promising detergent (CHAPS).

Affinity chromatography

Affinity chromatography matrices were hydrated, loaded into simple columns and equilibrated in buffer B. For the preparative 5'-AMP-agarose chromatography, six 5 ml columns were run in parallel, having a common outflow; 5 ml fractions were collected. A CHAPS-solubilized placental 25000 g pellet fraction (180 ml) was loaded (2.4 ml/min) on to the columns, which were then washed with 205 ml of buffer B and 45 ml of buffer B[0.25 M NaCl], and 11 β -HSD was eluted with 170 ml of buffer B[0.2 M NaCl + 1 mM NAD⁺], all at 3.5 ml/min. Fractions were placed on ice and rapidly assayed for 11 β -HSD enzyme activity. Fractions with abundant 11 β -HSD2 were pooled and concentrated (Amicon stirred cells; Centricon 10 concentrators; acetone precipitation).

Analytical work used 1 ml columns run manually (1.0–1.5 ml fractions). To obtain the highest purity of eluted 11 β -HSD an N-6-5'-AMP-agarose column was washed with buffer B (15 ml), buffer B[0.02 M NaCl + 0.4 mM NADH] (10 ml) and buffer B (15 ml), and then eluted with buffer B[0.125 M NaCl + 1 mM NAD⁺]. The flow rate was 420 μ l/min, decreasing to 200–300 μ l/min on elution. The increased purity was at the cost of lower yield and reproducibility than with the preparative protocol (above).

UV photoaffinity labelling and fluoroautoradiography

A portion of 467 μ l of tissue sample diluted in buffer C was added to wells of 24-well plates (diameter 15 mm) and maintained at 37 °C for 3 min. Addition of 60 μ l of 250 mM dithiothreitol, 48 μ l of 5 mM NAD⁺ (both in buffer C) and finally [³H]steroid (in 25 μ l of 10% ethanol/buffer C) completed the reaction, giving approx. 50 nM [³H]steroid in 600 μ l, unless otherwise

stated. In the experiments presented here (to minimize UV damage to protein), reactions were placed in UV light (312 nm transilluminator at a distance of 50 mm above plate; lid off) for 15 min at 37 °C. The procedure was easily scaled up. Similar labelling occurs at 254 nm and is even stronger at 0 °C or with longer UV exposure. However, all three of these variations also induce formation of minor bands below M_r 40000, and protein damage with prolonged 254 nm UV exposure will be more extensive.

Labelled samples were acetone-precipitated and resolved by SDS/PAGE [33] or 2-D electrophoresis. Finished gels, stained with Coomassie Blue to allow detection of major proteins and M_r standards, were processed for fluoroautoradiography in Entensify solutions and vacuum-dried before exposure to film.

2-D electrophoresis

2-D electrophoresis work involved running duplicate gels in parallel; usually one was silver stained (to visualize all the proteins), and the other stained with Coomassie Blue (to allow alignment between gels and to visualize SDS/PAGE protein standards) and processed for autoradiography. A set of 2-D electrophoresis protein standards was run under identical conditions on a third gel when new running parameters were used.

Conventional 2-D PAGE, i.e. isoelectric focusing (IEF) plus SDS/PAGE, used a variation of the method of O'Farrell [34]. 2-D non-equilibrium pH-gradient gel electrophoresis (NEPHGE) conditions were based on the methods of O'Farrell [35] and Witzmann [36], with variations of sample buffer and rod gel composition. Rod gels (170 mm \times 2.5 mm; length \times internal diameter) were cast from 9 M urea, 2 % Nonidet P40, 4 % acrylamide/bisacrylamide (19:1, w/w) and 3 % Pharmalytes (1.2 % pH 6–8 and 1.8 % pH 3–10), and polymerized with 32 μ l of 10 % ammonium persulphate and 27 μ l of *NNN'*-tetramethylethylenediamine (TEMED) per 20 ml of rod gel mixture. Optimal results (higher yield, better resolution), especially for basic hydrophobic proteins, were obtained with a dodecyl maltoside-based NEPHGE sample buffer: 5.71 g of urea, 154 mg of dithiothreitol, 4 ml of 10 % dodecyl maltoside, 750 μ l of Pharmalytes (300 μ l of pH 6–8; 450 μ l of pH 3–10) and 2.25 ml of HPLC-grade water, and adjusting the pH to 4.2 (by adding approx. 90 μ l of 5 M HCl and 160 μ l of HPLC-grade water). This buffer, which was a great improvement, is close to solidifying at room temperature and is melted, by warming to 30 °C, to adjust pH on preparation and on use. NEPHGE gels were run at 500 V for 2250 V \cdot h. After NEPHGE, rods were extruded into 10 ml of equilibration buffer for 7 min and either frozen or loaded directly on to second-dimension SDS/PAGE gels. Equilibration buffer (150 ml) consisted of 15 ml of β -mercaptoethanol, 9 g of SDS, 90 ml of HPLC-grade water, 5.6 ml of 0.075 % Bromophenol Blue and 37.5 ml of 4 \times Laemmli stacking buffer (0.4 % SDS, 0.5 M Tris/HCl, pH 6.8). For both IEF and NEPHGE the second dimension was run according to the resolving gel system of Laemmli [33] using 12.5 % gels (3.3 % cross-linker). Silver staining was by the method of Wray [37].

Concentrated protein from preparative AMP affinity chromatography runs was divided among several 1.5 mm-thick preparative 2-D gels. Precautions were taken to minimize protein N-terminal modifications: the second-dimension gel was pre-run in a Laemmli running buffer containing 0.5 % (v/v) freshly made 100 mM glutathione and then changed to running buffer with 0.1 % (v/v) 100 mM sodium thioglycolate [38]. These precautions were successful in allowing N-terminal sequence to be obtained for other proteins in our laboratory.

Protein blotting for sequencing

This was based on the CAPS [3-(cyclohexylamino)-1-propanesulphonic acid] buffer method of Matsudaira [39] and was performed from SDS/PAGE or second-dimension gels on to PVDF (Problott) membranes using a Bio-Rad Mini Trans-Blot apparatus. Sections of gels to be blotted were washed for exactly 30 s in CAPS transfer buffer (to allow shrinkage and removal of excess surface SDS). Electroblotting was for 75 min at 250 mA (90–110 V). After transfer, the PVDF sheets were stained (Amido Black), destained (water) and air dried, and desired bands/spots were cut out and stored at –70 °C.

Proteolytic digests of PVDF-blotted protein

These were based on the method of Fernandez et al. [40]. Membranes were cut into small pieces, blocked [0.2 % polyvinylpyrrolidone (average M_r 40000) in methanol for 45 min] and extensively washed in deionized HPLC-grade water. Autodigestion-resistant trypsin was added in AHT buffer (20 % acetonitrile, 1 % hydrogenated Triton X-100 in 100 mM Tris, pH 8) to a concentration of 1:20 (w/w) (trypsin/blotted protein), and digestion was continued for 24 h at 37 °C. Fragments were eluted using vigorous sonication and washes [AHT buffer, then 0.1 % (w/v) trifluoroacetic acid (TFA)] [40]. Eluted peptides were concentrated, removing the acetonitrile, and loaded as a 100 μ l sample on to an Applied Biosystems microbore HPLC system fitted with a C8 RP300 column (1 mm \times 250 mm; 7 μ m particle size) equilibrated in solvent A (0.1 % TFA/water). This was developed with a linear gradient of 0–100 % solvent B (0.08 % TFA in 80 % acetonitrile/water) over 45 min at 200 μ l/min. The spectrophotometric absorbance at 220 nm (A_{220}) of eluate allowed collection of peptide peaks. Control digests (trypsin with polyvinylpyrrolidone-blocked blank PVDF slices) were run in parallel to ensure identification of all peaks not due to the blotted protein substrate. A large peptide peak yielding no sequence which was thought to be the blocked N-terminal peptide was sub-digested in solution (50 mM sodium phosphate, pH 7.8) with *S. aureus* V8 protease at an estimated concentration of 20:1 (w/w, peptide/protease). Products were resolved on HPLC, as were those from a control (protease only) digest.

Amino acid sequencing

Peptide peaks selected for sequencing were pyridyl-ethylated [41], to allow detection of cysteine residues, and loaded into an Applied Biosystems 477A automated amino acid micro-sequencer.

RESULTS AND DISCUSSION

Subcellular fractionation

Human placental 11 β -HSD activity was well preserved during subcellular fractionation. Placental 11 β -HSD was clearly a membrane-associated protein (as < 2 % of activity remained in the cytosolic supernatant) and was most abundant in the 25000 *g* pellet, which contained over two-thirds of the total enzyme activity in under one-sixth of the total protein. Differential centrifugation of the 25000 *g* pellet did not produce any useful refinement. This fraction was used for further purification, and has been found to be very stable on storage at –70 °C, with no decline in activity in over 2 years.

Solubilization

Solubilization (i.e. remaining in supernatant after 1 h at 110000 *g*) of placental 11 β -HSD (from resuspended 25000 *g*

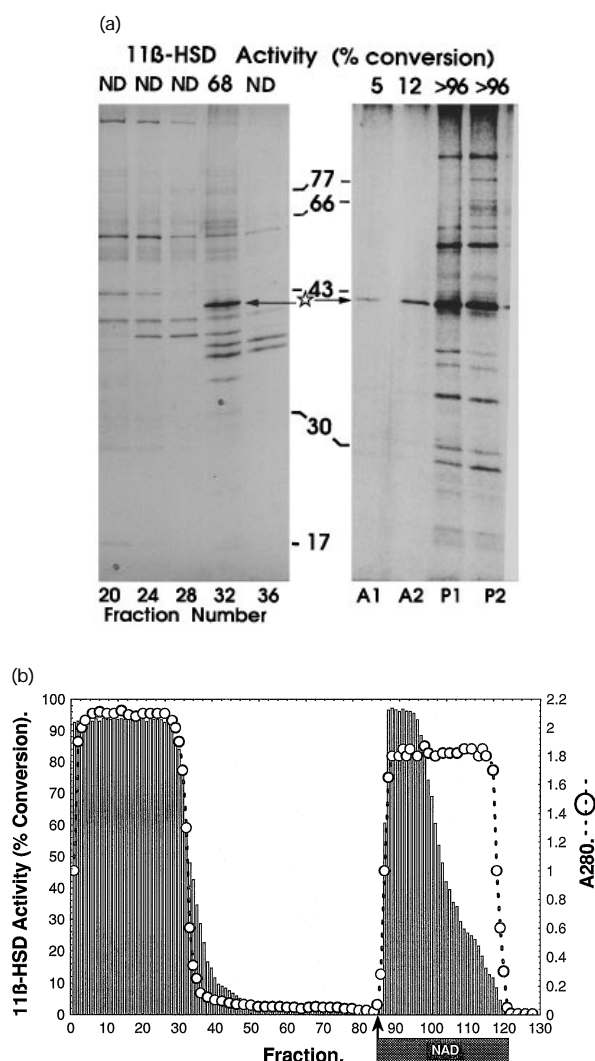


Figure 1 5'-AMP affinity chromatography

(a) SDS/12.5%-PAGE gels (silver stained) from 5'-AMP-agarose chromatography. 11 β -HSD2 activity (percentage conversion of 12 nM [3 H]corticosterone in 10 min) is indicated above the lanes; ND, not detected. The positions of 11 β -HSD2 protein (starred arrow) and of markers ($10^{-3} \times M_r$) are shown between the panels. Left panel, preliminary analytical study showing samples before, during and after harsh elution with NAD $^+$ (fractions 31–34); right panel, samples from two particularly clean analytical runs (A1 and A2) and pooled high-activity fractions from two typical preparative runs (P1 and P2), as described in the Experimental section. For analytical runs 190 μ l samples of fractions were used in standard 11 β -HSD assays and 1 ml samples (acetone precipitated) were used for SDS/PAGE; in preparative runs 50 μ l (assays) and 250 μ l (SDS/PAGE) samples were used. (b) Chromatographic profile of preparative-scale run showing 11 β -HSD2 activity (bars) in relation to A₂₈₀ (protein concentration or NAD $^+$ on elution) of fractions (5 ml) from the common outflow of six 5 ml AMP-agarose columns run in parallel. Fractions 1–36, loading; 37–86, washes; 87–120, elution with 1 mM NAD $^+$. The most active of these fractions are equivalent to samples P1 and P2 described above.

pellet) was attempted using 0–2.4 M NaCl. The ionic strength clearly affected 11 β -HSD activity before centrifugation (activity was highest with 0.3 M NaCl: 160% of that with no NaCl). As $\sim 99\%$ remained insoluble, solubilization was attempted with an extensive screen of detergents. Placental 11 β -HSD activity was inactivated by concentrations of detergents required to transform membrane proteins fully into micelles [detergent concentration \gg critical micelle concentration (CMC)]; however, limited though useful solubilization was achieved with gentle

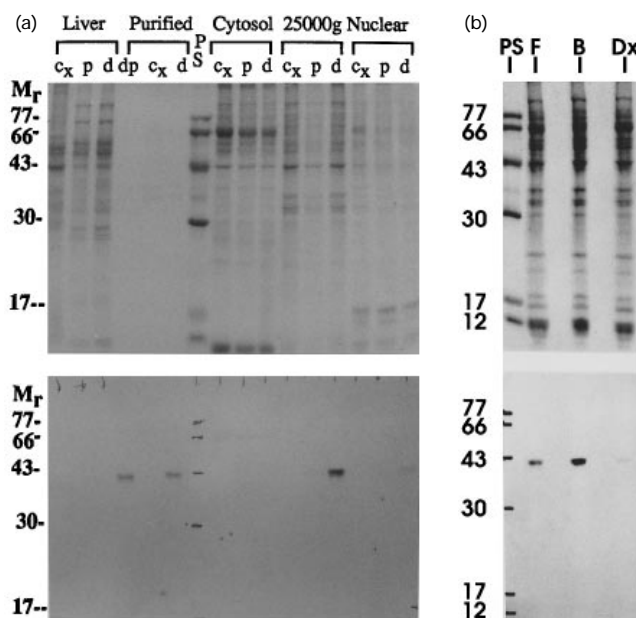


Figure 2 Native photoaffinity labelling of 11 β -HSD2

The bottom panels are autoradiographs of labelled protein samples run on the SDS/12.5%-PAGE gels shown in the top panels (stained with Coomassie Blue). $10^{-3} \times$ Apparent M_r of protein standards (PS) are indicated on the left side of the gels. (a) Triplicate samples of rat liver microsomes ('Liver'), AMP-chromatography-purified human placental 11 β -HSD ('Purified'; similar to fraction 32 in Figure 1) and three human placental subcellular fractions (Cytosol, 25000 g and Nuclear). All samples were photoaffinity labelled simultaneously with [3 H]corticosterone in the presence of 400 μ M NAD $^+$ (lanes d), 400 μ M NADP $^+$ (lanes p), both cofactors at 400 μ M (lanes dp) or both cofactors plus 50 μ M carbenoxolone (lanes c_x). A single protein band at 40 000- M_r is labelled in an NAD $^+$ -dependent, carbenoxolone-blockable fashion. (b) samples of human placental 25000 g pellet (mitochondria + heavy microsomes) photoaffinity labelled simultaneously in the presence of 400 μ M NAD $^+$ and [3 H]cortisol (F), [3 H]corticosterone (B) or [3 H]dexamethasone (Dx). Protein loading was $< 0.5 \mu$ g for 'Purified' lanes; otherwise it was $\sim 30 \mu$ g/lane.

conditions (detergent concentration $<$ CMC), being best with zwitterionic detergents with bile acid- or steroid-like head groups. CHAPS (4 mM) in combination with buffer C (which contains 0.3 M NaCl) was found to be optimal, solubilizing 40–45% of the 25 000 g pellet protein and 9.5% of its 11 β -HSD activity. On repeated re-extraction of the initially insoluble protein with 4 mM CHAPS, 13–20% of the original 11 β -HSD activity could be solubilized. Thus placental 11 β -HSD2 appears to be an integral membrane protein that requires factor(s) present in membrane subfractions of the placenta which are not satisfactorily substituted by a wide range of detergents.

Affinity chromatography

Since 11 β -HSD uses NAD $^+$ and glucocorticoids as substrates, columns with ligands that mimic these factors were screened. It was found that one matrix, N-6-linked 5'-AMP-agarose (Sigma; cat. no. A3019), bound substantial 11 β -HSD activity that could be eluted with cofactor (NAD $^+$ $>$ NADH $>$ 5'-AMP \gg NADP $^+$). Two other kinds of 5'-AMP-agarose [ribose hydroxyl- (Sigma A8895) and C-8-linked (Sigma A1271)] and two types of NAD $^+$ -agarose were ineffective, with a small percentage (2%) binding to the third type of NAD $^+$ -agarose available (linked at C-8; Sigma N1008), eluting specifically with NAD $^+$. 11 β -HSD activity did not bind to NADP $^+$ -agarose, cAMP-agarose or

dexamethasone-agarose (Sigma D4657; 0.5–1.0 μ mol/ml dexamethasone immobilized at C-17).

Preliminary studies with this N-6-linked 5'-AMP-agarose revealed it to be very useful for 11 β -HSD2 purification. Over a large number of running conditions, a band migrating at approx. M_r 40000 on SDS/PAGE was seen to co-segregate with the 11 β -HSD2 activity of the fractions (e.g. Figure 1a). Chromatography for highest purity (e.g. lanes A1 and A2, Figure 1a) suffered from a low yield of active 11 β -HSD.

The large-scale preparative conditions adopted (see the Experimental section and Figure 1b) reproducibly gave a 20-fold higher yield (35 %) at somewhat decreased, though still 1000-fold, purification (Figure 1a, lanes P1 and P2). The 40000- M_r band (starred) was the most prominent, but contamination persisted at various M_r values, notably several narrow bands very close to (especially above) 40000. These closely flanking bands indicated contaminating proteins that were likely to defeat further purification steps based solely on resolution by size (e.g. gel filtration). Accordingly it was decided to separate by size and charge using 2-D electrophoresis. As this would inevitably result in inactivation of 11 β -HSD2 activity, an alternative method of detecting the enzyme was required. This was provided by a novel affinity labelling method.

Affinity labelling

Kinetic studies of 11 β -HSD2 purified by AMP-agarose chromatography revealed a very low K_m for corticosterone (14 ± 1 nM) [27], and therefore affinity labelling with this steroid was attempted. As Figure 2 shows, a protein with all the expected characteristics of 11 β -HSD was uniquely photoaffinity labelled. This protein was M_r 40000 in size, and was present in the subcellular fractions along with 11 β -HSD activity [25000 g pellet 750 g pellet (nuclear/debris) \approx 110000 g pellet (light microsomes; not shown)], but absent from cytosol. The labelling was NAD $^{+}$ -dependent, independent of NADP $^{+}$ and blocked by carbenoxolone. Labelling of fractions after AMP-agarose chromatography showed the labelled protein exactly corresponded to 11 β -HSD activity, being present when 11 β -HSD was eluted with NAD $^{+}$ (e.g. Figure 1b, fractions 87–120) and in the flow-through (fractions 1–30), but absent from the inactive wash fractions. Moreover, the same protein could be affinity labelled with cortisol and dexamethasone (corticosterone \gg cortisol \gg dexamethasone; Figure 2b), but there was no labelling with aldosterone. This novel technique appears to result in completely selective labelling of placental 11 β -HSD by glucocorticoids, with a potency which parallels their affinities as substrates for the enzyme. Using this technique it was possible to track the 11 β -HSD2 protein after activity had been lost, so allowing final purification by 2-D electrophoresis.

The affinity labelling technique developed here is unusually straightforward and specific for 11 β -HSD2. Photoaffinity labelling is more commonly described for receptors and binding proteins, and a few such procedures report labelling by unmodified steroid compounds acting as the ligand (e.g. GR by dexamethasone), but even in such cases these 'common' ligands lack the desired specificity and are replaced by compounds developed to allow high specificity (e.g. RU26988 and RU28362 for exchange assays/photoaffinity labelling specific for the GR [42,43]). Affinity labelling of enzymes is much less common by photoactivation, and is more often achieved using a specifically designed activatable compound containing a reactive functional group (usually electrophilic, e.g. bromoacetate) which is attacked by an activating substituent (usually a nucleophilic side chain of an amino acid) in the enzyme's active site (e.g. 3-methoxyoestril

16-(bromo[2- 14 C]acetate) labelling of homogeneous placental 17 β -HSD type 1 at two histidines in its active site [44]). Less commonly, enzymes can be photoaffinity labelled by precisely chosen synthetic compounds which, when photoactivated, develop short-lived, highly reactive substituents which label the target enzyme if a suitable amino acid residue is nearby (e.g. UV-induced labelling by 19-nortestosterone acetate of purified Δ^5 -3-ketosteroid isomerase from *Pseudomonas testosteroni* [45]). What makes the labelling technique described here particularly unusual is that it can use a physiological substrate in a crude tissue extract containing thousands of different proteins (in contrast to the two examples given above, which used pure enzyme), and labels the enzyme for that substrate with such specificity that analysis reveals only a single spot on 2-D electrophoresis. To our knowledge, the only other study describing photoaffinity labelling of a steroid-metabolizing enzyme by what may be a natural substrate is the labelling of Pseudomonas Δ^5 -3-ketosteroid isomerase with testosterone [45]. This, however, is a less avid label than synthetic 19-nortestosterone acetate. Though the parallels are intriguing, that study [45] utilized pure enzyme and \sim 500-fold higher steroid concentrations than were employed here for 11 β -HSD2 labelling.

Several findings suggest that the affinity labelling procedure described above occurs within the steroid binding pocket of the active site. Firstly, the affinity labelling is NAD $^{+}$ -dependent, as is 11 β -HSD2 activity. There are a few reports of cofactor-dependent affinity labelling of other dehydrogenases. NAD $^{+}$ -dependent lactate dehydrogenase is affinity labelled, in the presence of NAD $^{+}$, by bromopyruvate on the major catalytic residue (histidine-195) [46]. Secondly, carbenoxolone, an 11 β -HSD2 inhibitor, blocks labelling, as does a 1000-fold excess of unlabelled corticosterone. Thirdly, the rank order of potency for glucocorticoid labelling correlates with the substrate affinities of human placental 11 β -HSD2.

Finally, the glucocorticoid groups most likely to participate in covalent photoaffinity labelling of 11 β -HSD2 are the carbonyl group at C-3 and the C-11 position itself. Labelling via the C-20 carbonyl appears less likely as it is expected to be less photoexcitable, and covalent attachment of the C-20–C-21 grouping (following the common C-17–C-20 cleavage) would not attach any radioactivity, as the steroids are tritiated at C-1, -2, -6 and -7 only. The C-3 carbonyl would be highly photoactivated to produce both alkoxy radicals and reactive ketenes; both of these would react relatively indiscriminately [47], potentially with almost any closely approximated amino acid, but especially with reactive cysteine, lysine, histidine or tyrosine residues. The photoreactive state of C-11 is harder to anticipate, but is likely to be greatly heightened in the active site where it will make the transition to a C-11 carbonyl, a photoexcitable group in its own right.

2-D electrophoresis

Fractions containing enzyme eluted from AMP-agarose were affinity labelled with [3 H]corticosterone and then subjected to conventional 2-D electrophoresis (IEF plus SDS/PAGE). After prolonged exposure, gel autoradiography remained negative. Accordingly, NEPHGE gels were run. A strong signal, indicating the presence of a single, affinity-labelled, protein spot of basic pI (\sim 9.1) and apparent M_r 40000, was seen on autoradiography (Figures 3a and 3b). It was notable that this was accompanied by other proteins close to 40000- M_r which were not affinity labelled. NEPHGE conditions were optimized (Figure 3) to allow preparative-scale gels with substantially increased yield and reduced smearing of the affinity-labelled protein, which was

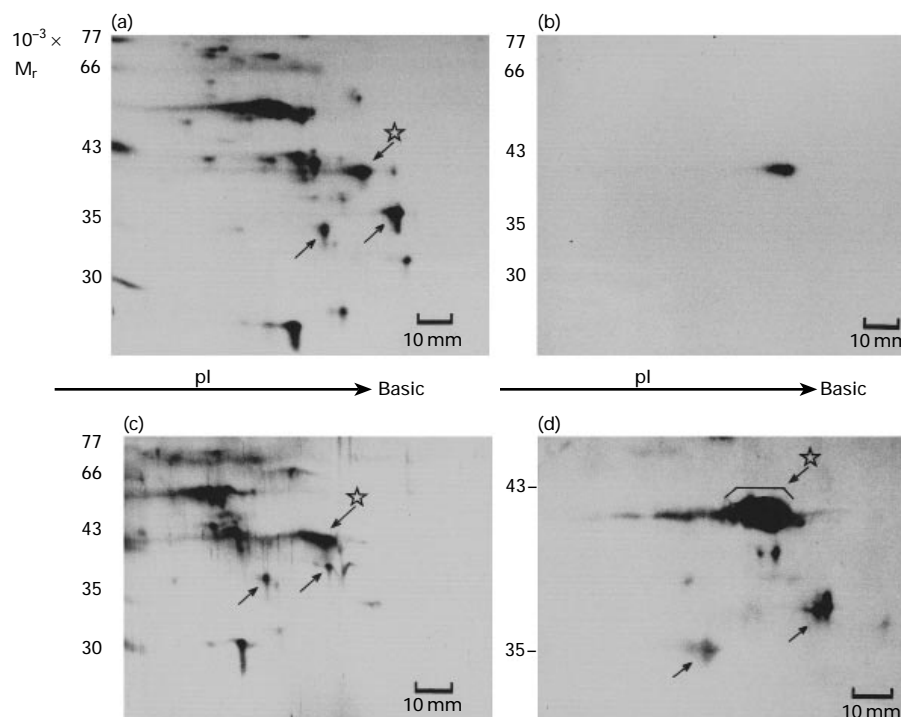


Figure 3 Identification and preparative 2-D electrophoresis of homogeneous 11 β -HSD2

Panels (a), (c) and (d) show silver-stained second-dimension gels of NEPHGE 2-D electrophoresis (running to basic pH as indicated). 11 β -HSD2 (starred arrow) and two protein contaminants (upward-pointing arrows) are indicated to assist orientation. Panel (b) shows an autoradiograph of a gel run in parallel, identical to the one in (a), but stained with Coomassie Blue and processed for autoradiography. (a) and (b) NEPHGE analysis, with an even pH gradient (3–10; see the Experimental section) showing that the protein band(s) at 40 000- M_r resolve into multiple protein spots, of which 11 β -HSD2 (clearly identified by autoradiography; b) is one of the most abundant and basic in pI. (c) NEPHGE analysis with the pH gradient expanded at pH 6.5–8.0 to allow better horizontal separation of 11 β -HSD2 from nearby proteins at M_r 40 000–43 000. (d) Optimized preparative-scale 2-D electrophoresis for 11 β -HSD2 with dodecyl-maltoside-based buffer (greatly reducing streaking) and running the second dimension further to increase the vertical resolution. Note that the 10 mm scales refer to actual gels.

otherwise a major problem. The optimized procedure allowed preparation of over 40 μ g of homogeneous 11 β -HSD2 protein, which was blotted on to PVDF (Problott) membranes.

Several findings suggest that the 11 β -HSD2 membrane environment has polar aspects. Thus moderate ionic strength stimulates activity, the most hydrophobic lipid-like detergents (e.g. Tween 20) are not the most useful for preserving 11 β -HSD2 activity, and the 11 β -HSD2 protein has a very high pI (> 9). The very basic pI (9.1) is surprising. Proteins this basic are unusual, often being ribosomal or associated with nucleic acid (chromatin or RNA). Rarely are they intrinsic membrane proteins, and this combination suggests interactions of 11 β -HSD2 beyond NAD⁺ and steroids, perhaps with other membrane proteins or charged lipid. Thus it may well be that interactions of this nature (especially with phospholipid head groups) may be involved in stabilizing the enzyme, as occurs with the related NAD⁺-dependent short-chain alcohol dehydrogenase (SCAD) enzyme 3-hydroxybutyrate dehydrogenase [48]. We have not investigated this further, as development of the affinity labelling technique allowed purification of 11 β -HSD2 to homogeneity, when activity was not maintained.

Amino acid sequence

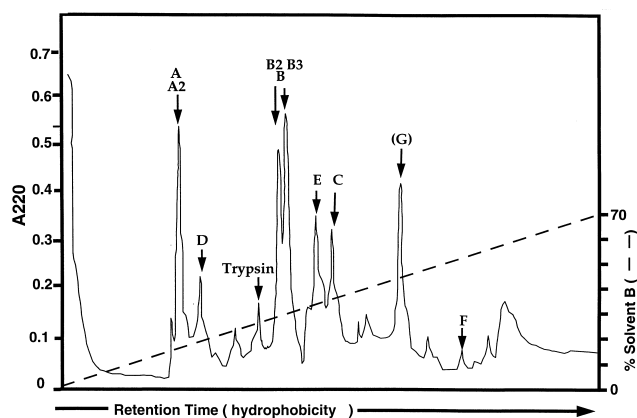
PVDF blots were stained with Amido Black and the 40 000- M_r target protein accurately excised. Sequencing of PVDF pieces revealed that homogeneous 11 β -HSD2 was N-terminally blocked. This is unlikely to be an artifact of the purification as, on sequencing, an amino acid signal was completely absent

(rather than just low), and we have obtained high-yield N-terminal sequence from other proteins blotted from gels in the same fashion. *In situ* tryptic digestion of approx. 30 μ g of 11 β -HSD2 protein produced multiple peptides and several of these were sequenced, yielding in total over 100 residues of amino acid sequence (Figure 4 and Table 1). Peptide G was blocked. This was sub-digested with *S. aureus* V8 protease, yielding a sequencable daughter peptide.

All peptide sequence obtained was unique, confirming 11 β -HSD2 as a novel protein. This is the first directly determined 11 β -HSD2 peptide sequence from any source. Direct amino acid sequencing provides information about amino acid order and post-translational modification, and avoids some pitfalls of cDNA-predicted amino acid sequencing, such as the use of rare codons (e.g. selenocysteine). There is one potential N-linked glycosylation site (NLS; peptide B) which in the native protein, at least in human placenta, appears to be largely free of glycosylation, as the amino acid sequence yield did not drop across this asparagine residue. Peptide C contains a classical SCAD motif (boxed in Table 1) indicating that 11 β -HSD2 belongs to this enzyme superfamily. There was one clear 'blank' cycle in the middle of peptide C (cycle 15); it is possible that a modified amino acid naturally occurs here. Further discussion of the sequence is presented in the accompanying paper [30].

Overall purification procedure

Table 2 summarizes the steps in the purification procedure described that allowed isolation of homogeneous 11 β -HSD2



The solid line indicates the elution of peptide peaks (detected by A_{220} , highest peaks corresponding to ~ 500 pmol). The elution positions of trypsin and peaks from which the peptide sequence was obtained (G obtained by V8 sub-digest) are indicated. The digest was loaded on to the HPLC RP300 column equilibrated in solvent A (0.1% TFA in water) and developed with a linear gradient (broken line) of 0–100% solvent B (0.08% TFA and 80% acetonitrile in water) over 45 min at 200 μ l/min.

protein from human placenta (> 16000-fold purification) and direct determination of over 100 residues of its internal amino acid sequence. In an accompanying paper [30], the amino acid

sequence derived has been used to clone a full-length 11 β -HSD2 cDNA from a human placental library which, on transfection into mammalian cells, expresses 11 β -HSD2 activity and an approx. 40000- M_r protein which can be affinity labelled by the method described above.

The primary objective of this work was to accomplish 11β -HSD2 purification. We feel that it also allows conclusions to be drawn which may shed some light on the form of the enzyme's active site and kinetic mechanism. The striking specificity of the photoaffinity labelling of 11β -HSD2 by corticosterone and cortisol, in the presence of NAD^+ , demonstrates that 11β -HSD2 exhibits an unusually high, receptor-like, affinity for these glucocorticoids, which may come close to that of GR or MR. This finding underlies the physiological role of 11β -HSD2 in binding, and inactivating, glucocorticoids with high avidity before or in competition with GR and MR, so regulating glucocorticoid access to receptors. 11β -HSD2 is probably not the same as the $\sim 45000\text{-}M_r$ 'low-affinity' ($K_d \sim 100\text{ nM}$) dexamethasone binding proteins identified in studies on male rat liver [49], as it differs in localization, hierarchical order of binding affinities and possibly Mg^{2+} -dependence of binding (11β -HSD2 labelling occurs in EDTA-containing buffers). However, 11β -HSD2 does resemble another, incompletely characterized, receptor/binding protein for glucocorticoids: the type 3 corticosteroid (corticosterone) receptor [50,51]. Thus the rank order of affinities determined in binding studies of these 'type 3 sites'

The amino acid and yield (in pmol) obtained on sequencing 11b-HSD2 peptides A–G are given by cycle number. Peptide B was sequenced twice (using a second sample from tryptic digestion of purified 11b-HSD2). Cycle C(15) produced no residue signal. Cycles F(5), F(8), F(13) and C(6) produced two residues with similar yield, making assignment difficult. Cycle A(5) is definitely W, but the yield imprecise. Note that the boxed sequence in peptide C is a classical SCAD motif, indicating the likely active site.

[illegible]

Table 2 Summary of purification of 11 β -HSD2

Purification using 10 placentas yielded $\sim 43 \mu\text{g}$ of $\sim 16\,000$ -fold-purified 11 β -HSD2. Affinity labelling of tracer amounts of 11 β -HSD2 after AMP-agarose chromatography allowed a means of assessing yields beyond this stage. 11 β -HSD2 peptide recovery (following *in situ* tryptic digestion, elution from PVDF and microbore HPLC) was generally lower with increasing peptide hydrophobicity (see Figure 4). Activity was measured as nmol/min per mg of protein.

Purification step	Protein	Yield (%)		Purification (fold)		Activity (nmol/min per mg)	Recovery of radioactive tracer (%)
		Step	Cumulative	Step	Cumulative		
Homogenate	46.8 g	—	100	—	—	0.025	—
Subcellular fractionation	5.22 g	67	67	6	6	0.150	—
Solubilization	1.85 g	13	8.7	0.367	2.2	0.055	—
AMP-agarose	640 μg	36	3.15	1040	2290	57.3	—
2-D gel	43 μg	47	1.47	> 7	> 16 000	—	100
Blot	30 μg	70	(1.05)	—	—	—	70.2
Eluted peptides	$\sim 520 \text{ pmol}$ ($\sim 21 \mu\text{g}$)	70*	(0.75)	—	—	—	49.8
Initial sequencing yield	445 pmol*	85	—	—	—	—	—

* Best yield.

[50,51] is consistent with the order of potency of the four steroids used to affinity label 11 β -HSD2 (Figure 2). 'Type 3 sites' are often regarded as cytosolic, which 11 β -HSD2 is not. However, the work originally defining them (in rat kidney) largely used supernatants from centrifugation at 30 000 *g* for 30 min, which is very similar to the 25 000 *g*/40 min fractionation that we have used. Supernatant from such a spin will probably have approx. 95 % of protein from the cytosol, but the remaining 5 % will be rich in protein from light microsomes and contain abundant 11 β -HSD2. Recent work on isolated renal cortical collecting duct cells characterizes a similar binding site in whole cells (where endogenous NAD⁺ may facilitate binding), which may be the renal 11 β -HSD2 isoform [51]. The characteristics of placental 11 β -HSD2 are thus clearly similar to the 'type 3 sites' in these reports. In the accompanying paper [30] this matter is addressed further by examining the affinities of recombinant 11 β -HSD2. However, at present it is not settled whether 'type 3 sites' and 11 β -HSD2 are the same entity.

The affinity chromatography data provide some clues to the reaction order and nature of the active site of placental 11 β -HSD2. It is likely that 11 β -HSD2 binding to N-6-5'-AMP-agarose was via an interaction at the cofactor binding site. 5'-AMP is a 'half molecule' of NAD⁺ (nicotinamide comprising the remainder), and instances of N-6-5'-AMP affinity matrices interacting with the cofactor site of NAD⁺-dependent dehydrogenases are well documented [52]. We have shown the rank order of potency by which free cofactors elute bound human placental 11 β -HSD2 activity from N-6-5'-AMP-agarose to be NAD⁺ > NADH > 5'-AMP \gg NADP⁺ (with 10-fold more 5'-AMP than NAD⁺ required for elution) [27]. Thus for free cofactor the binding site prefers NAD⁺, so appearing like a NAD⁺ cofactor site as opposed to a 5'-AMP allosteric site. When the cofactor is on the affinity matrix and not free, however, the rank order changes to N-6-5'-AMP (yield over 35 %) > C-8-NAD⁺ (yield ~ 2 %) > N-6-NAD⁺ = C-8-5'-AMP = NADP⁺ (yield = 0). Clearly the attached spacer arms make a considerable difference, sterically hindering NAD⁺ from binding more strongly than 5'-AMP (as occurs with free cofactors). This may be a limitation of the particular affinity matrix products used or simply be because the larger size of NAD⁺ makes its binding more sensitive to steric hindrance. A further possibility is that NAD⁺ may cause a conformational change on binding (this quite frequently occurs with dehydrogenases), whereas 5'-AMP, filling only a half site, does not. Thus steric interference by the new conformation

prevents 11 β -HSD2 binding to N-6-NAD⁺, but some limited binding is then possible with C-8-NAD⁺ (which has the spacer attachment rotated > 90° relative to N-6, both coming from the 5'-AMP half of NAD⁺). 5'-AMP, causing no such conformational change, binds well when N-6-linked, but not at all when C-8-linked. The 5'-AMP affinity chromatography results suggest that NAD⁺ can bind first to 11 β -HSD2 (explaining both binding and elution with NAD⁺). The NAD⁺-dependence of glucocorticoid affinity labelling suggests that glucocorticoid cannot gain full access to the steroid binding pocket unless NAD⁺ has bound first. Affinity chromatography with dexamethasone-agarose was unsuccessful under a range of elution conditions. The finding that under similar conditions 11 β -HSD2 is bound well by N-6-5'-AMP agarose, but not at all by dexamethasone-agarose (though dexamethasone is a substrate), may possibly also reflect a 'cofactor first' binding order. By analogy with other dehydrogenases (e.g. lactate dehydrogenase [46]), these findings collectively suggest that a compulsorily ordered ternary complex mechanism may operate for 11 β -HSD2, with NAD⁺ binding first. Further studies of these matters will assist in mapping the active site and in the design of drugs specifically to inhibit, or possibly constitutively activate, 11 β -HSD2.

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